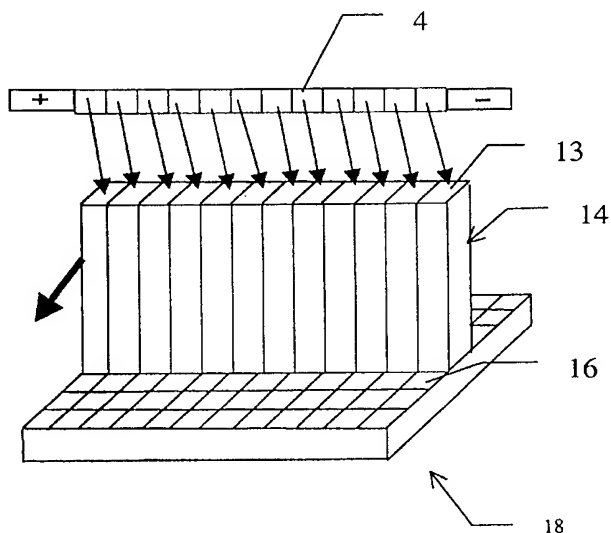




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/SE99/01674 (22) International Filing Date: 23 September 1999 (23.09.99) (30) Priority Data: 9803224-6 23 September 1998 (23.09.98) SE (71) Applicant (for all designated States except US): AMERSHAM PHARMACIA BIOTECH AB [SE/SE]; Björkgatan 30, S-751 84 Uppsala (SE). (72) Inventor; and (75) Inventor/Applicant (for US only): LAURIN, Ylva [SE/SE]; Tegelgatan 6, S-752 38 Uppsala (SE). (74) Agents: ROLLINS, Anthony, J. et al.; Nycomed Amersham plc, Amersham Labs, White Lion Road, Amersham, Bucks HP7 9LL (GB).		(81) Designated States: JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>

(54) Title: METHOD FOR SEPARATION OF MACROMOLECULES



## (57) Abstract

The invention relates to a method and a system, suitable for automation, for the, preferably preparative, separation of amphoteric macromolecules, e.g. proteins or peptides, wherein said method comprises the steps of: (a) subjecting said mixture of macromolecules to isoelectric focusing in liquid media; (b) collecting samples from step (a), said samples containing macromolecules separated on basis of isoelectric point, and transferring each sample to a channel (13) accommodating medium for electrophoresis, said channel (13) being part of a composite body (14); (c) subjecting said samples, contained in the channels (13) in said composite body (14), to electrophoresis; and (d) allowing electrophoresis to proceed until macromolecules are eluted from said medium for electrophoresis, and collecting fractions of the samples containing macromolecules.

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## METHOD FOR SEPARATION OF MACROMOLECULES

### TECHNICAL FIELD

5 The present invention relates to methods for the separation, in particular, the preparative separation of amphoteric macromolecules by two-dimensional electrophoresis, whereby isoelectric focusing in liquid media is combined with electrophoresis, and subsequent electroelution to liquid media. The invention further relates to a system for separation of amphoteric macromolecules, comprising an  
10 isoelectric focusing unit, a composite body having channels accommodating the electrophoresis medium and an elution plate for use in said methods.

### BACKGROUND ART

15 The principles for two-dimensional electrophoresis are known in the art. This technique has normally involved first subjecting a mixture of molecules to isoelectric focusing on a gel where a pH gradient has been established. As the molecules traverses the pH gradient they reach at some point a pH corresponding to their  
20 respective isoelectric point and stop migrating. Traditionally, this separation has been performed in a capillary gel, which then has been placed on top of a slab gel in order to subject the sample to SDS-PAGE, whereby the molecules are separated according to their molecular weights. In order to further process the separated  
25 molecules, additional handling steps are required, for example detecting the spots, cutting the gel pieces out of the slab gel and extraction of the material from the gel. A problem with this method is low recovery rates. Another problem with this method is that it is a largely manual procedure which requires skill to perform it and which is time-consuming to perform.

30 Isoelectric focusing can also be performed in liquid media, as disclosed in US 4,971,670. A multi-chambered isoelectric focusing unit (IsoPrime™ unit) is commercially available. With this unit, a pH-graduated set of buffered polyacrylamide

- 2 -

membranes separates a series of chambers through which a sample circulates. Each membrane defines a specified step in a pH gradient. Each chamber encompasses a pH (or pI) range determined by the pH values of the membranes that bound it. During electrophoresis, a protein passes through membranes into successive chambers until it reaches the chamber encompassing its pI. The protein remains focused in this chamber and can subsequently be recovered from the chamber. This process is intended to separate and purify one amphoteric compound from a flow of compound. It is not suitable for separating a plurality of compounds that are to be analysed and which have the same isoelectric point as all compounds having the same isoelectric point end up in the same chamber. They then have to be extracted from the chamber and further separated by some other means.

A composite body having channels suitable for accommodating gels for electrophoresis is disclosed in WO 97/37216. Said composite body comprises a wall structure made from integrally formed element having a plurality of parallel longitudinally extending channels, said channels accommodating chemical medium or media suitable for carrying out a test, analysis or reaction procedure in situ in the channels.

However, the presently known methods for separating mixtures of amphoteric macromolecules often requires multiple manual steps and are difficult to automate. In addition, the known methods are not optimised to prepare samples suitable for direct use in downstream applications, such as e.g. mass spectrometry.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic upper view of a device suitable for the first-dimension isoelectric focusing step.

Figure 2 is a schematic view of a composite body suitable for second-dimension electrophoresis and a collecting plate.

Figure 3 is a side view of the lower buffer reservoir of the collecting plate.

Figure 4 is a side view of a channel filled with electrophoresis medium together with  
5 the upper buffer container and the collecting plate.

## DISCLOSURE OF THE INVENTION

10 The present invention provides a method for separating mixtures of amphoteric macromolecules which avoids the above mentioned drawbacks and is designed for automation. With the use of the methods according to the invention, samples are obtained in solution, ready for downstream processing by means of e.g. mass spectrometry or other characterisation methods.

15 Consequently, in a first aspect the invention provides a method for separating amphoteric macromolecules, e.g. proteins or peptides, comprising the steps  
(a) subjecting said mixture of macromolecules to isoelectric focusing in liquid media;  
(b) collecting samples from step (a), said samples containing macromolecules  
20 separated on basis of isoelectric point, and transferring each sample to a channel accommodating medium for electrophoresis, said channel being part of a composite body;  
(c) subjecting said samples, contained in the channels in said composite body, to electrophoresis; and  
25 (d) allowing electrophoresis to proceed until macromolecules are eluted from said medium for electrophoresis, and collecting fractions of the samples containing macromolecules.

The method according to the invention will be further described in the following  
30 sections, with reference to the mentioned steps (a) to (d):

*(a) The isoelectric focusing step*

The isoelectric focusing step can conveniently be performed in a unit having a series of chambers separated by membranes, each chamber encompassing a pH range determined by said membranes. Such units are known in the art from e.g. US 4,971,670. However, it will be understood that according to the present invention, said units and separation chambers can be considerably smaller than those known in the art. In the method according to the invention, a separation chamber can e.g. conveniently have a volume from about 50 to about 500  $\mu$ l, preferably about 100  $\mu$ l.

In a preferred embodiment of the invention, the isoelectric focusing unit can be as schematically shown in Figure 1. Such a unit, which in itself is a further aspect of the invention, can have the form and size of an ordinary microtitre plate (1) (normally about 15 x 85 x 125 mm). The plate (1) comprises a buffer chamber (2) at each short end and the anode (+) and cathode (-) can be placed or integrated in the buffer chambers. The plate has 8 rows and 12 columns of separation chambers (4), which are separated from the buffer chambers (2) by semi-permeable membranes (6) which have a suitable cut-off point (e.g. 2000 Da) to make them non-permeable to the macromolecules to be separated.

For isoelectric focusing, the biological sample, which normally contains from about 0.1 to 5 mg protein, is applied in one or more of the separation chambers (4), which are divided by membranes (8), preferably buffered polyacrylamide membranes, which each have a defined pH, thus defining a pH gradient, such as pH 3 -10 or 4-7, along a row of separation chambers. The separation chambers (4) are filled with a buffer which normally will contain urea, a non-ionic detergent, carrier ampholytes and a reduction agent. A suitable buffer could e.g. contain 8 M urea; 4% CHAPS or 2% Triton-X100; 0.5 to 2% Pharmalyte® (Amersham Pharmacia Biotech, Uppsala, Sweden); and 60 mM dithiothreitol (DTT).

The 8 rows of separation chambers (4) are divided by walls (10) which run in the direction of the current, i.e. perpendicular to the pH-defined membranes (8), and which are non-permeable to the biological macromolecules to be separated.

Consequently, several distinct samples can be subjected to isoelectric focusing on one single plate without any risk that molecules will cross over from one row to another.

5 The isoelectric focusing is normally allowed to proceed for from 5.000 to 50.000 volt hours, depending on sample load and pH interval. During the isoelectric focusing step, each macromolecules in the biological sample migrates over the membranes (8) in the rows of separation chambers (4), until it reaches the chamber (4) which encompasses its pI. The molecule remains focused in this chamber (4).

10 *(b) Transfer to the electrophoresis step*

After isoelectric focusing, samples are collected, either manually or, preferably automatically using computer controlled multi-channel pipettes, by extracting the fluid  
15 from each of the separation chambers (4) in turn and transferring it to the channels (13) in the composite body (14) for electrophoresis. As illustrated in Figure 2, the, for example, twelve samples collected from one row of separation chambers (4) can conveniently be transferred to the corresponding twelve channels (13) in a composite electrophoresis unit (14). Alternatively 8 samples from a column of eight separation  
20 chambers (4) can be transferred to 8 channels (13) in a composite electrophoresis unit (14). If necessary, suitable additives, such as SDS (sodium dodecyl sulphate), colour (e.g. brome phenol blue, BFB) and/or glycerol, can be added to the samples  
*(c) The electrophoresis step*

25 The composite body (14) used for electrophoresis comprises a plurality of parallel longitudinally extending channels (13) suitable for accommodating electrophoresis media. In the present context the term "channels" is intended to include capillaries, as well as channels of a larger dimension. In the example shown in figure 2, the composite body (14) preferably has the same number of channels (13) as the  
30 number of separation chambers (4) in a row of separation chambers in plate (1), i.e. composite body (14) has twelve channels (13). However, composite body (14) can conceivably have any number of channels.

The composite body (14) can comprise e.g. parallel capillaries (13) that are attached together in a single line and regularly spaced. Alternatively, the composite body (14) can comprise a wall structure with longitudinally extending channels (13) in a single line. The channels can have circular, square or rectangular cross-sections, although other cross-sectional shapes are possible.

Said wall structure is preferably extruded from a synthetic material, such as glass or a plastic material, such as polypropylene. A suitable material is a commercially available polypropylene material, in the form of semi-rigid sheets, known as Correx™.

The chemical medium for electrophoresis may be uniform throughout the channels (13) or may vary in strength or concentration in a regular or other predetermined way across the array of channels. Different media may be accommodated in different channels. Also, the chemical medium may be arranged to vary, e.g. in concentration, along the length of the channel. The electrophoresis medium can be e.g. agarose, polyacrylamide, or another matrix forming medium.

During electrophoresis, the composite body (14) is placed into contact with a container (26) in which the cathode (30) is placed or integrated, and which contains the upper electrophoresis buffer. Such a container (26) can e.g. have the form of a collar (26), which is tightly fitted to the upper part of the composite body (14) (Figure 4). The buffer or buffers to be used in the electrophoresis step can be obtained by well known methods, e.g. as described by Laemmli (1970) Nature (London) 227, 680; or by Garfin, D.R., Methods Enzymol. Vol. 182, 425.

The electrophoresis step can be performed under with or without the addition of SDS (sodium dodecyl sulphate) to the samples and/or buffer. SDS is added to obtain denaturing conditions in standard SDS-PAGE, for the separation of macromolecules based on molecular weight, while addition of SDS has to be avoided e.g. for investigations of protein conformation, proteins interactions or protein–nucleic acid interactions.

*(d) The collection step*

When the electric field is applied in the electrophoresis arrangement, the negatively charged molecules migrate towards the anode (22) and can consequently be eluted and collected from the electrophoresis channels (13) by letting electrophoresis proceed until the samples have reached the end of the gels. At this stage, the macromolecules subject to electrophoresis have been further separated on e.g. the basis of molecular weight.

In a preferred form of the invention, the composite electrophoresis unit is dimensioned to allow for elution of samples from the channels (13) directly into wells (16) of a collecting plate (18) which can have the form and size of an ordinary microtitre plate. The collecting plate has a number of rows of wells (16) where the number of wells (16) in each row is the same as the number of channels (13) in the composite body (14). It will be understood that for this purpose, each of the electrophoresis channels (13) will have a protruding part (32) that will make sample delivery into discrete wells (16) on the collecting plate (18) possible.

Rather than having a solid bottom, as with ordinary microtitre plates, the collecting plate (18) is preferably fitted with a filter or semi-permeable membrane (20) which covers the open bottom of each well (16) and which has a suitable cut-off point (e.g. 2000 Da) to make it non-permeable to the macromolecules to be separated. This is schematically illustrated in Figure 3, which is a partial side view of the lower buffer chamber (24), in which the anode (22) is placed or integrated.

Figure 4 further schematically illustrates a suitable arrangement for electrophoresis and sample collection, including a channel (13) integrated in a composite electrophoresis unit (14) and harbouring electrophoresis medium. The composite unit is in contact with a container (26) in which the cathode (30) is placed or integrated, and which contains the upper electrophoresis buffer. The sample subject to electrophoresis is loaded in the space (28) between the top of the electrophoresis gel

and the top of the channel. The lower portion (32) of the channel (13) is protruding into the well (16) on the collecting plate (18). The well (16) contains lower electrophoresis buffer, in order to create continuous contact between the upper and lower electrophoresis buffers via the electrophoresis medium, so that an electric field  
5 can be applied between the anode (22) and cathode (30).

During elution of separated macromolecules, the composite body (14) comprising the electrophoresis channels (13) can conveniently be moved stepwise (as illustrated by the arrow in Figure 2), manually or, preferably, automatically since a large number of  
10 fractions need to be collected to obtain good separation, over the collecting plate (18), so that the lower portions (32) of the channels (13) protrude into a new row of wells (16). During the stepwise movement the composite body is lifted up, for example by a robot (not shown), from the collecting plate (18), thereby temporarily stopping the elution. The elution automatically recommences when the lower  
15 portions (32) of the channels (13) are lowered back into contact with the electrophoresis medium. Consequently, elution takes place for a time period with the composite body (14) placed over the collecting plate (18) so that samples are eluted from each of the channels (13) into the corresponding well (16) on the collecting plate (18). After the defined time period, the composite body (14) is lifted, moved to  
20 the next row of wells (16), and then lowered so that elution is continued. This procedure is repeated until elution of the separated macromolecules is complete. It is of course possible for the composite body to remain still and the collecting plate to be moved instead.

25 When electrophoresis and collection of samples are completed, the liquid samples can be transferred, by manual or, preferably, automatic procedures, from the wells (16) for storage or for direct use in downstream applications, such as mass spectrometry etc.

30 In another important aspect, the invention provides a system for separating amphoteric macromolecules comprising (a) means for isoelectric focusing, comprising a unit having a series of chambers (4) separated by membranes, each

chamber encompassing a pH range determined by said membranes; (b) a composite body having channels accommodating gels for electrophoresis; and (c) a collecting plate. It will be understood that said system is adapted for automatically performing the methods according to the invention, and that the various parts of said system consequently have the preferred features described above.

Consequently, said isoelectric focusing unit preferably has at least one series of chambers separated by membranes, each chamber encompassing a pH range determined by said membranes, and has essentially the dimensions of a microtitre plate. Preferably it comprises a plurality of rows of separation chambers, said rows being divided by walls that are non-permeable to the macromolecules to be separated.

Preferably, said collecting plate essentially has the dimensions of a microtitre plate and said composite body has preferably dimensions that allow elution from the channels directly into the wells of such a collecting plate.

The collecting plate preferably comprises a semi-permeable membrane covering the bottom of each well, said membrane being non-permeable to the macromolecules to be collected.

## CLAIMS

1. A method for separation, especially preparative separation, of amphoteric macromolecules, comprising the steps of:
  - 5 (a) subjecting said mixture of macromolecules to isoelectric focusing in liquid media;
  - (b) collecting samples from step (a), said samples containing macromolecules separated on basis of isoelectric point, and transferring each sample to a channel (13) accommodating medium for electrophoresis, said channel  
10 being part of a composite body (14);
  - (c) subjecting said samples, contained in the channels (13) in said composite body (14), to electrophoresis; and
  - (d) allowing electrophoresis to proceed until macromolecules are eluted from said medium for electrophoresis, and collecting fractions of the samples  
15 containing macromolecules.
2. The method according to claim 1 wherein the isoelectric focusing step is performed in a unit (1) having at least one series of separation chambers (4) separated by membranes (8), each chamber encompassing a pH range  
20 determined by said membranes (8).
3. The method according to claim 2 wherein said unit (1) essentially has the dimensions of a microtitre plate.
- 25 4. The method according to claim 2 or 3 wherein said unit (1) comprises a plurality of rows of separation chambers (4), said rows being divided by walls (10) which are non-permeable to the macromolecules to be separated.
5. The method according to any one of claims 1 to 4 wherein said composite body  
30 (14) is in contact with a container (26) harbouring a cathode (30) and electrophoresis buffer.

6. The method according to any one of claims 1 to 5 wherein samples from the electrophoresis channels (13) are eluted directly into wells (16) on a collecting plate (18).
- 5 7. The method according to claim 6 wherein said collecting plate (18) essentially has the dimensions of a microtitre plate, and said composite body (14) has dimensions which allows for elution from the channels (13) directly into the wells (16) of said collecting plate (18).
- 10 8. The method according to claim 6 or 7 wherein said collecting plate (18) comprises a semi-permeable membrane (20) covering the bottom of each well (16), said membrane (20) being non-permeable to the macromolecules to be collected.
- 15 9. An isoelectric focusing unit for use in a method according to any of claims 1-8, having at least one series of separation chambers (4) separated by membranes (8), each separation chamber (4) encompassing a pH range determined by said membranes (8), characterised in that said isoelectric focusing unit essentially has the dimensions of a microtitre plate.
- 20 10. An isoelectric focusing unit according to claim 9, comprising a plurality of rows of said series of separation chambers (4), said rows being divided by walls (6) which are non-permeable to the macromolecules to be separated.
- 25 11. A system for separating amphoteric macromolecules in accordance with the method according to any of claims 1-8 comprising
  - (a) means for isoelectric focusing, comprising a unit having a series of separation chambers (4) separated by membranes (8), each separation chamber (4) encompassing a pH range determined by said membranes (8);
  - 30 (b) a composite body (14) having channels (13) accommodating gels for electrophoresis; and
  - (c) a collecting plate (18).

12. A system according to claim 11 wherein said composite body (14) is in contact with a container (26) harbouring a cathode (30) and electrophoresis buffer.
- 5 13. A system according to any one of claims 11 to 12 wherein said collecting plate (18) essentially has the dimensions of a microtitre plate and said composite body (14) has dimensions which allows for elution from the channels (13) directly into the wells (16) of a said collecting plate (18).
- 10 14. The system according to claim 13 wherein said collecting plate (18) comprises a semi-permeable membrane (20) covering the bottom of each well 16), said membrane (20) being non-permeable to the macromolecules to be collected.

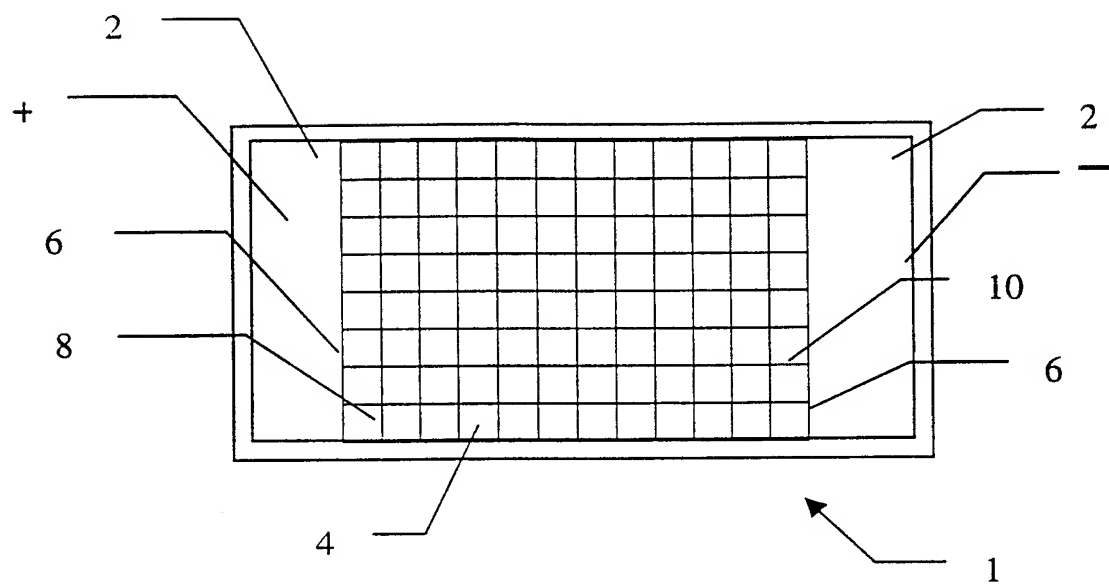


Fig. 1

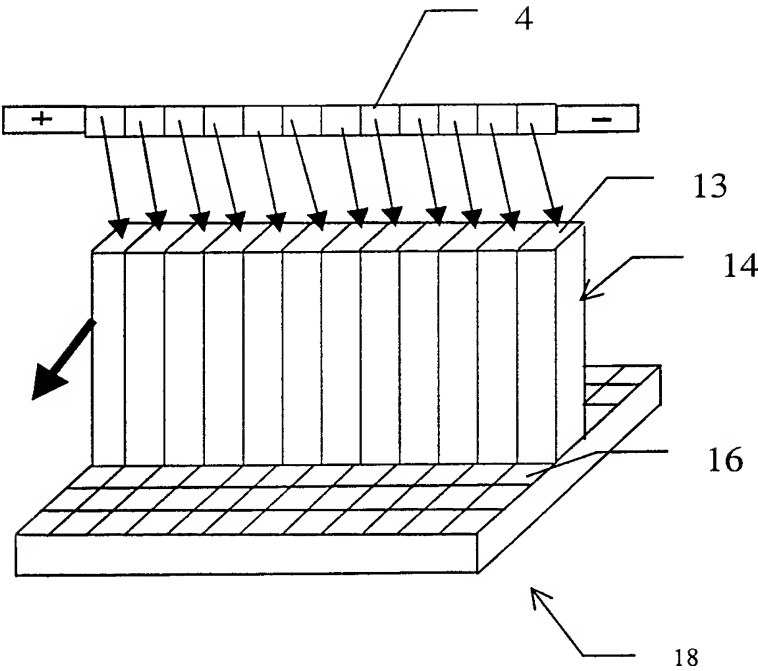


Fig. 2

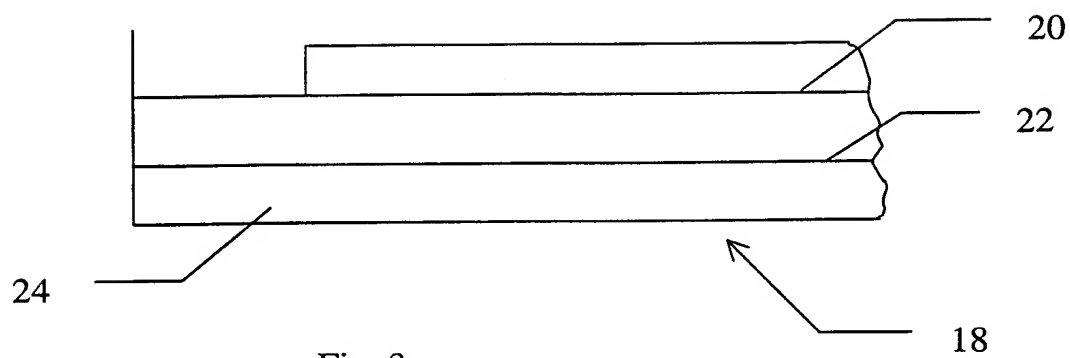


Fig. 3

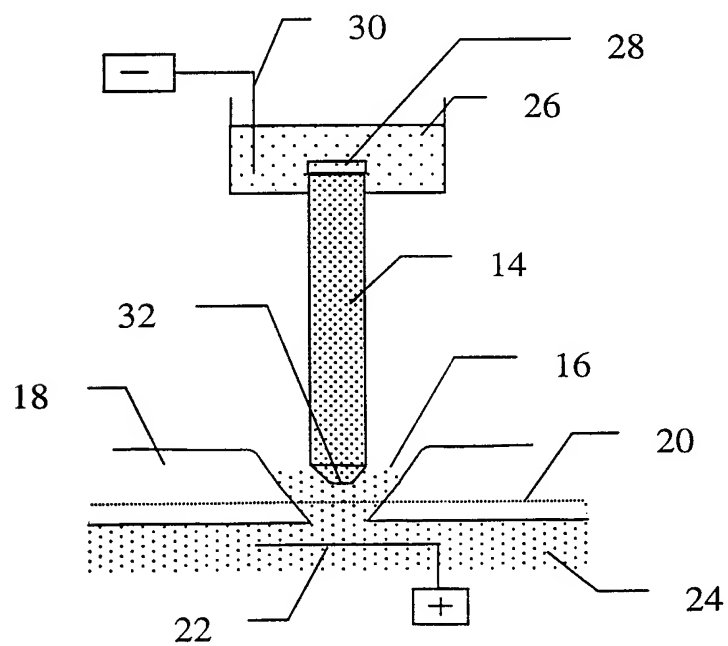


Fig. 4

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/01674

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## A. CLASSIFICATION OF SUBJECT MATTER

IPC7: G01N 27/447, C07K 1/28

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5773645 A (DENIS FRANCOIS HOCHSTRASSER), 30 June 1998 (30.06.98), abstract --	1,5-8,13-15
Y	WO 9737216 A1 (DEAR, PAUL ET AL), 9 October 1997 (09.10.97) --	1,5-8,13-15
A	US 4971670 A (DANIEL M. FAUPEL ET AL), 20 November 1990 (20.11.90), column 8, line 43 - line 53, figure A, claim 10 --	9-10
A	WO 9825135 A1 (MACQUARIE RESEARCH LTD.), 11 June 1998 (11.06.98), abstract -- -----	1-15

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance  
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 "O" document referring to an oral disclosure, use, exhibition or other means  
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

20 January 2000

Date of mailing of the international search report

29 -01- 2000

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**INTERNATIONAL SEARCH REPORT**International application No.  
**PCT/SE99/01674****Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/SE99/01674

1. Claims 1-8 and 11-15 relates to a method for separating macromolecules with two-dimensional electrophoreses.
2. Claims 9-10 relates to an isoelectric focusing unit with at least one series of chambers separated by membranes.

The technical features according to the first invention relate to a method for separating macromolecules with two-dimensional electrophoreses, while the special technical features of the second invention relate to an isoelectric focusing unit with at least one series of chambers separated by membranes for use in a method according to the first invention. These groups of inventions are not so linked as to form a single general inventive concept. The isoelectric focusing unit is not considered to be specially designed for carrying out the method claimed in claim 1, because the contribution it makes over prior art does not correspond to the contribution the method makes over prior art.

As all claims could be searched without effort justifying an additional fee, this authority did not invite payment of any additional fee.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

02/12/99

International application No.  
PCT/SE 99/01674

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 9825135 A1	11/06/98	AU 5184998 A AU P0403496 D	29/06/98 00/00/00